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(54) Title: METHOD FOR MODULATING SELF-DESTRUCTION OF CYTOLYTIC LYMPHOCYTES

(57) Abstract

Cytotoxic thymus derived lymphocytes, in the absence of other cells, are destroyed by exposing them to their cognate peptides (those that are presented by class I major histocompatibility complex (MHC-I) proteins for recognition by a T cell's antigen-specific receptors). The destruction is proportional to peptide concentration; it can be specifically prevented by a second peptide that competes with the cognate peptide for presentation by the CTL's MHC-I protein; and it is probably due primarily to self-destruction of individual CTLs (suicide) rather than to the destruction of some CTLs by others of the same clone in the same culture (fratricide). The method can be used *in vivo*, or *in vitro*, using cognate peptides to eliminate selected CTL clones that cause pathological cell destruction, as in some autoimmune diseases and some viral infections.

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METHOD FOR MODULATING SELF-DESTRUCTION OF CYTOLYTIC LYMPHOCYTES

Background of the Invention

The present invention is a method for inducing destruction of cytotoxic thymus derived (T) lymphocytes (CTLs) by exposing them to their cognate peptides.

The Government has rights in this invention pursuant to grant number NIH-R35-CA-42504 awarded by the National Institute of Health.

B cells react with antigens that are either in solution or on a cell surface, but a T cell characteristically reacts only with antigen on the surface of another cell. This restriction arises because the antigenic structures recognized by T cells consist of binary, noncovalent complexes formed by the association of peptide fragments of protein antigens with histocompatibility proteins encoded by genes in the major histocompatibility complex (MHC). The MHC proteins, and thus the complexes they form with antigenic peptides, are found only on cell surfaces.

There are two classes of MHC proteins, MHC-I and MHC-II. In association with peptides, each is recognized selectively by one of the two principal sets of T cells. One of the principal sets has the CD4 glycoprotein on the cell surface ($CD4^+$ T cells): these cells recognize peptide/MHC-II complexes and generally function as helper cells (i.e., they enhance the responses of B cells and some other cells). The other principal set, constituting around forty percent of mature T cells, express the CD8 glycoprotein on their surface ($CD8^+$ T cells) and recognize peptide/MHC-I complexes. The $CD8^+$ T cells generally function as cytotoxic T lymphocytes (CTLs) and lyse target cells whose peptide/MHC-I complexes they recognize.

Under normal circumstances it is likely that the peptide moiety of the peptide/MHC-I complexes arises within the target cell itself from proteolysis of newly synthesized proteins. However, short synthetic peptides (typically 10-25 amino acids in length) that resemble the natural peptide moiety are evidently also effective in creating the antigenic complexes, since cells that express an appropriate MHC-I protein become specific target cells, susceptible to lysis, simply upon incubating them with an appropriate peptide, termed the cognate peptide.

Cognate peptides are defined as those peptides that are recognized in association with particular MHC-I proteins by the antigen-specific receptors on appropriate T cells.

Normally, CTLs and their target cells express the same MHC-I proteins (i.e., they are syngeneic). Hence, when a synthetic cognate peptide is added to a mixture of CTLs and syngeneic target cells, the peptide should associate not only with an appropriate MHC-I protein on the target cell but on the CTL as well. Nevertheless, in the standard 4-hour assay used to measure cytolysis it has been repeatedly observed with many different peptides, CTLs, and syngeneic target cells that the addition of a cognate peptide induces the CTLs to lyse the target cells but apparently not to lyse themselves. A possible explanation for the apparent sparing of the CTLs in this assay stems from current ideas of the mechanism which lead CTLs to kill their targets. According to an extensively studied mechanism, when a CTL adheres to a target cell whose antigen's structure it recognizes, the CTL is stimulated to release cytotoxic granules into the synapse-like cleft between it and the target cell. When isolated from CTLs, these granules readily lyse a wide

variety of normal and transformed cells, but CD8 T cells, and especially cloned CTL cell lines, are relatively resistant.

Clinical and experimental observations show that individuals sometimes produce antibodies and T cells that react with antigens of their own cells and tissues. These exceptions to the principles of self-tolerance often appear to be responsible for disease. There are several proposed mechanisms for autoimmunity, including altered forms of self-antigens which acquire novel epitopes; altered distribution of those self-antigens, such as sperm and eye antigens, that the immune system normally does not encounter; antigenic mimicry between epitopes of bacterial and viral proteins and self-proteins; chronic viral infections which introduce novel epitopes on infected cells; and defective production of suppressor T cells which allows the emergence of anti-self ("autoreactive") T cells. The epitopes of many bacterial and viral proteins, consisting of short linear amino acid sequences (e.g., nine to twelve amino acids in length), bear some resemblance to sequences in host proteins. Hence immune responses to the microbial epitopes can sometimes cross-react with self-antigens. Examples include a human cytomegalovirus protein (IE2) and a human class II-MHC protein (DR); a poliovirus protein (VP2) and acetylcholine receptor; a protein (P24) of the human immunodeficiency virus (HIV) and a sequence in the human IgG constant region; and a protein (P3) of measles virus and myelin basic protein. Monoclonal antibodies to coxsackie virus B4 cross-react with heart muscle; this virus has also been identified in individuals with myocarditis. Many times autoimmune disorders are diagnosed on the basis of the presence of antibodies which cross-react with self-proteins, yet it is unclear whether the antibodies are the agents causing the disease or only symptomatic of the disease. Considerable evidence

strongly suggests that cell mediated immunity, due to T cells, plays at least as great a role in pathogenicity.

CTLs are also responsible for extensive cell damage in certain viral infections. A classic example is seen in mice infected with the lymphocytic choriomeningitis virus (LCMV). LCMV infection of immunologically unresponsive mice, such as nude or newborn mice, causes a chronic widespread infection but the mice remain healthy. If these infected mice are injected with anti-LCMV CTLs, they die. Human hepatitis virus may be another example. It has been suggested that virus infection of liver cells would be relatively benign were it not for the destruction of these cells by CTLs that recognize hepatitis virus peptides, in association with liver cell MHC-I proteins.

At present, therapy for autoimmune diseases is non-specific in that it is not directed at the underlying cause, but rather at suppressing the activity of the entire immune system, or one of its major components. Immunosuppressants which are currently in use include glucocorticoid, methotrexate, aziothiaprin, cyclophosphamide, non-steroidal anti-inflammatory agents, antimalarials, cyclosporin A, and other relatively non-specific agents. Usage and dosage of these drugs are dictated by the disease manifestations. Glucocorticoid, for example, are used in high dosages to treat some neurologic complications of systemic lupus erythematosus (SLE). Both azathioprine and cyclophosphamide are used as an attempt to halt or reverse renal damage. Limiting side effects are common for all of the immunosuppressants. At high doses, effective at controlling disease, they expose patients to the risk of infections with opportunistic microbes; at low doses, minimizing this risk, they are only marginally effective. Other limitations derive from other toxic side effects. For

example, the dose of cyclophosphamide is generally regulated, and limited by toxicity to hematopoiesis.

It is therefore an object of the present invention to provide a method for eliminating T cells reactive with particular self or other antigens.

It is a further object of the present invention to provide a method for limiting autoimmune disease.

It is still another object of the present invention to provide a method for identifying cognate peptides.

It is a further another object of the present invention to facilitate the use of peptides as vaccines.

Summary of the Invention

In the primary embodiment, a method is disclosed for eliminating particular cytotoxic T lymphocytes (CTLs) by exposing them to their cognate peptides. The CTLs self-destruct in proportion to the concentration of cognate peptides. The method can be used *in vitro* or *in vivo*. Timing of administration is crucial since the CTLs must be activated and cytolytically competent.

The method can be used *in vivo* to treat autoimmune disorders characterized by CTLs which recognize known peptide sequences. In the preferred form, the cognate peptide is administered in a drug delivery matrix, such as a biodegradable polymer that releases peptide over a specific period of time.

In another embodiment, the method is used to enhance vaccination against the cognate peptide, where the response is measured by production of CTLs recognizing the peptide.

Brief Description of the Drawings

Figure 1A is a cross-sectional schematic view of an activated CTL. Figure 1B is an expanded view of the CTL surface showing the interaction on a single cell between the cell's MHC-I protein (consisting of a heavy chain subunit and ß-microglobulin), in association with a cognate peptide (Pc), and the cell's antigen-specific T cell receptor, a heterodimer of an α and a ß subunit.

Figures 2A, B, C and D are cell counts of cloned $CD8^+$ CTLs that were cultured in the absence or presence of cognate peptides at various concentrations.

Panel A shows the number of cells per ml when a cognate peptide (OVA-2) was added to an anti-ovalbumin CTL clone (2H7) that had been stimulated for the preceding three days by incubating it with specific target cells (irradiated EG7-OVA cells, which are EL-4 tumor cells that express a transfected ovalbumin gene). Three days after adding the cognate peptide the number of cells per ml had decreased in proportion to the peptide's (OVA2) concentration: at 20 μg/ml the number of surviving cells were around 1/10th as large as the number per ml in the absence of peptide (O).

Panel B shows the number of cells per ml one day later (day 4); the decrease was even more striking.

Panel C shows the number of cells per ml with a tumor-specific CTL clone (12D3), one day after transferring the target cell-stimulated CTLs into fresh medium; even 0.01 μg/ml of the cognate peptide (T1) led to a striking decrease in the number of surviving cells within one day.

Panel D shows the number of cells per ml when another anti-ovalbumin CTL clone, 3G12, was stimulated for 3 days with the EG6-

OVA cells and then separated from the latter by dilution into fresh medium to which the OVA-2 peptide was added.

Figure 3 is a comparison of the cytolytic activity of CTLs in three clones: 4G3, 12D3, and IE1, each maintained for three days in the presence or absence of various peptides. The column at the left shows their cytolytic activity when no peptide had been added. The column second to the left is the cytolytic activity when the cognate peptide (OVA-2) for clone 4G3 was preincubated with the CTLs; the third column is the cytolytic activity when the cognate peptide (T1) for clone 12D3 was preincubated with the CTLs; and the fourth column is the cytolytic activity when the cognate peptide (pp89) for clone IE1 was preincubated with the CTLs. In each case only the cognate peptide eliminated the activity of the corresponding clone. (▲) EL4 cells + OVA-2, 5 μg/ml; (△) EL4 cells alone; (○) P815 cells + T1 peptide, 1 μg/ml; (■) P815 cells + pp89, 1 μg/ml; (□) P815 cells.

Figure 4 is graph comparing the number of cells of an anti-ovalbumin CTL clone, 4G3, remaining after incubation with its cognate peptide, OVA-2, alone and in combination with an inhibiting peptide, pp89, showing that the extent of inhibition varied with the blocking peptide concentration.

Since pp89 specifically blocks the sensitization of conventional target cells by OVA-2, the ability of pp89 to block OVA-2 induced self-destruction of anti-ovalbumin CTLs means that when these CTLs self-destruct, they recognize OVA-2 on themselves in the same way that they recognize this peptide on conventional target cells.

Figures 5, 6 and 7 are comparisons of the cytolytic activity of spleen cells from primed and naive animals at effector:target cell ratios

of 1:1 to 1:100, measured as percent cell death (specific ^{51}Cr-release) on three ^{51}Cr-labeled target cells.

Figure 5, panels A1 and A2, shows the cytolytic activity of spleen cells that were derived from naive mice and stimulated one or more times *in vitro* with irradiated EG7-OVA cells (C), and then tested after five days (assay #1, in A1) and after 24 days (assay #2, in A2) for cytolytic activity on three target cells: 1) EG7-OVA (○), 2) EL-4 cells that were sensitized by incubating them with the OVA-2 peptide (termed EL-4 + OVA-2 targets) (▲), and 3) EL-4 cells alone (termed nonsensitized targets) (△). Greater lysis of the EL-4 + OVA-2 targets than of nonsensitized EL-4 targets provided a measure of OVA-2-specific CTLs. The cells from naive mice (A1 and A2) had no cytolytic activity against OVA-2, although they eventually developed some low-level cytolytic activity against some epitopes of EL-4 cells (cf. panels A1 and A2). Anti-OVA-2 activity in spleen cells obtained from mice that had been primed with EG7-OVA cells is shown in panels B1 (assayed after 5 days) and B2 (assayed after 24 days). Anti-OVA-2 activity in spleen cells obtained from mice that had been primed with one or two injections of ovalbumin is shown in panels C1 and D1, respectively (each assayed at 5 days). Some anti-OVA-2 activity in spleen cells obtained from mice that had been primed twice with the OVA-2 peptide alone is shown in panel D2.

Figure 6 examines the effect of stimulating spleen cells over a period of five days in culture to determine if an injection of the cognate peptide (OVA-2) into mice could decrease the effect of priming the CTLs *in vivo*. The peptide was injected once into each of several immunized mice, from 1 to 5 days after the final priming injection (with ovalbumin). Mice that had received no peptide at all, or had been

injected with the peptide on day 1 or day 5 after the final priming injection had indistinguishable levels of OVA-2-specific CTL activity. However, injection of the peptide on day 2 resulted in the elimination of OVA-2-specific cytolytic activity; the remaining activity was essentially the same on all three target cells, demonstrating that the remaining active CTLs were specific for epitopes of the EL-4 cells but not for OVA-2. Injection of peptide on day 3 or day 4 resulted in a reduction but not in a complete elimination of anti-OVA-2 CTL activity.

Figure 7 also shows that primed CTLs can be inactivated *in vivo*. Mice were primed by two injections of EG7-OVA cells (C), 30 days apart, and injected with the OVA-2 peptide (P) once (A1 and A2) or twice (B1 and B2). When their spleen cells were analyzed after 5 days (assay #1) or 24 days (assay #2), in culture, it was clear that the second injection of OVA-2 (B#1 and B#2), or perhaps the two injections of peptide, had eliminated the primed anti-OVA-2 CTLs *in vivo*.

Detailed Description of the Invention

Based on the discovery that cognate peptides can induce self-destruction of cytolytic T lymphocytes, a method to selectively destroy T lymphocyte clones *in vitro* and *in vivo* has been developed. The method also increases the probability that an individual can be immunized against short peptides, which generally do not elicit an immunogenic response characterized by CTLs recognizing the peptide.

T cells recognize linear sequences of amino acids in small, 10-20 residue peptide fragments of antigens in conjunction with one of two main classes of cell-surface glycoprotein of the major histocompatibility complex (MHC): either class I (MHC-I) or class II (MHC-II) proteins. The short peptides recognized (in association with MHC protein) by the

T cells are referred to as "cognate peptides". $CD8^+$ cells recognize antigen fragments in conjunction with MHC-I, whereas $CD4^+$ cells recognize them in conjunction with MHC-II. Only special antigen-presenting cells (APC) express MHC-II, so $CD4^+$ T cells react only with APC and are referred to as "helper T cells". However, MHC-I is expressed on the surface of all cells except erythrocytes so $CD8^+$ cells can react with virtually any cell in the body. Most $CD8^+$ T cells destroy cells whose surface antigens they recognize and are therefore known as cytotoxic T lymphocytes or CTLs.

Recognition of a target cell's antigen stimulates the CTL not only to release its cytolytic components but also to proliferate. Accordingly, CTLs are usually maintained in culture by periodically incubating them with target cells having the appropriate surface antigen, either because these cells express the corresponding genomic gene, or are infected with an appropriate virus, or are "pulsed" by briefly incubating them with synthetic cognate peptides and then washed to remove unbound peptides. However, attempts to maintain CTLs in culture simply by periodically adding cognate synthetic peptides to a mixture of these cells and syngeneic target cells led instead to a decrease in the number of CD8 CTLs. Accordingly, the effects of synthetic peptides on CTL clones was examined, and was found in all cases to induce CTLs to destroy themselves. Because the peptide-induced self-destruction also occurs *in vivo*, this effect helps explain why immunization with synthetic peptides often fails to elicit the formation of CD8 CTLs. The findings also suggest a mechanism for eliminating *in vivo* potentially autoreactive CD8 CTLs that can recognize their own self-peptide/MHC-I complexes. This amounts to a method for eliminating particular CTL clones to control the damage

they can cause in some autoimmune diseases and in some viral infections.

As demonstrated by the following observations, CTLs can be destroyed by exposing them under appropriate conditions to their cognate peptides. The destruction is proportional to peptide concentration and can be blocked by a second peptide that interferes with MHC-presentation of the cognate peptide. The findings suggest that CTLs destroy themselves when their antigen-specific T cell receptors recognize a cognate peptide in association with their own MHC-I protein. The destruction appears to be due primarily to the self-destruction of individual CTLs (suicide), rather than to the destruction of some CTLs by other CTLs of the same clone (fratricide). The destruction is also dependent on the state of activation of the CTLS, and on the time of exposure to the peptide. In a conventional reaction between a CTL and a target cell (whether another CTL or a different cell-type altogether) the two cells adhere to each other as "conjugates" only transiently. The conjugates persist only briefly (as little as six minutes, and usually much less than an hour); their transience is evident from the low frequency of conjugates in CTL-target cell mixtures, and from the well-known migration of CTLs from one target cell to another. In contrast, when a single CTL serves as its own target, by virtue of presenting a cognate peptide via its own MHC-I molecules to its own antigen specific T cell receptor (TcR) molecules, the interactions are likely to be long-lasting, so long as the peptide is present. The chronicity of this self-attack is probably responsible for the cell's eventual self-destruction.

When a CTL engages in peptide-induced self-destruction it has two disparate roles. In serving as a target cell, it probably does not

have to be in an activated state; but in serving as a cytolytically active cell it must be activated. (CTLs are normally cytolytically effective for only a few days after being stimulated by interaction with conventional target cells or with antibodies to the TcR-CD3 receptor complex.) It appears that the cognate peptide will only induce self-destruction of CTLs when they have been recently stimulated and are cytolytically competent.

This process is more clearly understood by reference to Figure 1. Figure 1A is an activated CTL. Figure 1B is an expanded view of the CTL surface showing a specific interaction between the complex formed by a MHC-I protein (consisting of a heavy chain subunit and ß-microglobulin) with a cognate peptide (Pc), and the cell's antigen-specific receptor. This interaction triggers the self-destruction of the CTL.

The method described herein has been demonstrated to be applicable with mouse CTLs, which, like all cells, express MHC-I proteins. It cannot occur with normal mouse $CD4^+$ T cells, because these cells generally do not express MHC-II proteins and these proteins are required for cognate peptide-presentation to $CD4^+$ T cells. However, activated human $CD4^+$ T cells do express MHC-II proteins. Some of these cells are also cytolytic for the cells they recognize. Since human CD8+ T cells are entirely analogous to mouse CD8+ T cells, the method demonstrated here with mouse CD8+ CTLs should be applicable to human cytolytic CD4+ T cells as well as to human CD8+ CTLs.

Many peptides, when injected into mice, stimulate the development of $CD4^+$ T helper cells, but similar peptides, injected similarly, often fail to elicit the development of $CD8^+$ CTLs. In

contrast, target cells that express endogenously derived peptides in association with surface MHC-I are potent immunogens for $CD8^+$ T cells. Although it is possible that exogenous peptides are presented much less effectively than endogenously derived peptides to precursor $CD8^+$ cells, perhaps because of large differences in the surface density on target cells of the relevant peptide/MHC-I binary complexes, the examples described here support the proposition that the cognate peptide induces self-destruction of activated CTLs *in vivo*, just as it does in culture. As a result, it is likely that when mice are immunized by repeated injections of a cognate peptide, any $CD8^+$ T cells that are successfully primed by one injection are at risk of undergoing self-destruction if a subsequent injection of the same peptide achieves an adequate concentration in the immediate environment of the responding cells when they have become cytolytically competent.

A method has been developed based on this analysis to prime CTLs without destruction of cytolytically competent cells. The example demonstrates that competent anti-ovalbumin $CD8^+$ CTLs can be elicited by using widely spaced injections of the OVA-2 peptide (Fig. 7, panel D2): under these conditions cells primed by the first injection would not have been cytolytically active when the peptide was injected a second time, leading to their further priming and eventual successful restimulation with EG7-OVA cells.

Given the great range in specificity of CTLs and their enormous capacity to destroy target cells, any CTLs that can recognize self-peptides in association with self-MHC-I proteins are obviously potentially pathogenic. It is thought that the precursors of such cells are normally eliminated as they mature in the thymus. However, the findings described herein suggest that if any of these precursors escape

the thymus they would self-destruct in the periphery as they become activated in response to the self-peptide/MHC-I complexes they themselves generate. Presumably, it is the self-peptides produced by other cells of the same individual, but not by the CTLs themselves, that are involved in autoimmune pathology and which can be destroyed using the method of the present invention, once the cognate peptides are known and appropriately administered. Elimination of critical cytolytic T cell clones, while leaving the immune system as a whole intact, suggests that cognate peptides, once identified and properly administered, could contribute to the realization of a long-term goal of immune therapy.

In summary, a method has been developed based on the discovery that cognate peptides can induce self-destruction of a CTL if the cell is cytolytically activated and the peptide is provided in an effective concentration and for a sufficient time. The method is useful in selectively destroying CTL clones specific for autoantigens. To treat an autoimmune disorder, between microgram and milligram quantities, preferably between one and 200 milligrams, of any given peptide of between 5 and 50 amino acids, preferably between 10 and 20 amino acids, is administered to an adult human in a suitable pharmaceutical vehicle, such as a polymeric drug delivery device, alone or in combination with an immunostimulant such as the lymphokines interleukin 1, 2 or 4, to the patient. Since better results are achieved when cells are maximally activated, the patient's CTLs should be stimulated prior to administration of the peptide. Peptides can be administered alone or in combination with other peptides.

Few of the peptides involved in autoimmune disorders are known. Known antigenic proteins include a portion of the receptor for

thyroid stimulating hormone, which is the antigen in Graves disease, and a portion of the acetylcholine receptor, which is the antigen in myasthenia gravis. The method of the present invention, used as a screening technique, suggests how more of these peptides can be identified. Cells can be isolated from affected tissue and cell surface MHC proteins extracted and fractionated to yield MHC-associated peptide adducts. CTLs collected from the same patient can then be exposed to the fractionated peptide adducts. Self-peptides which induce self-destruction of the CTLs are presumed to be involved in the pathogenesis of the disorder.

Peptides are made synthetically using standard techniques and equipment for synthesis of short amino acid sequences. They are administered by slow intravenous drip or adsorbed to, or with, an acceptable pharmaceutical carrier, or, more preferably, in a polymeric drug delivery device. Methods and materials for making controlled release polymeric matrices are known to those skilled in the art. The preferred polymers are ethylenevinyl acetate or poly(lactic acid-glycolic acid). Other polymers include polyanhydrides, poly(ortho esters), polyesters and other biodegradable polymers, including proteins. See, for example, U.S. Patent Nos. 4,906,474 to Langer, et al., 4,391,797 to Folkman, et al., 4,069,307 to Higuchi, et al., 4,675,189 to Kent, et al., and 4,925,673 to Steiner, et al., the teachings of which are incorporated herein. Other methods and materials are also known to those skilled in the art for encapsulating peptides in, or within, polymeric matrices which are shaped and/or degrade to release the peptide over a period of time. The peptide can also be encapsulated within one type of matrix coated with a second matrix containing peptide, each matrix having different rates of degradation, such that

peptide is released initially from the outer coating, then released from the inner matrix after a period of time. Materials are available that have degradation rates ranging from a few hours to years, that are biocompatible, and inert to the encapsulated peptides. Other materials can also be incorporated which interact with the peptides to enhance the immunogenicity and/or stability.

For destruction of specific CTL clones, it is desirable to have release extending over a period of several days, although exposure of cells to peptide for periods as short as six hours results in killing. Changes in cell shape are observed after only one hour of exposure to peptide. CTLs generally have around 50,000 to 100,000 MHC molecules per cell. It is believed that it is only necessary to occupy between 1% and 10% of these molecules to obtain self-lysis of CTLs. This is probably achieved at between nanogram to microgram/ml concentrations of peptide. The effective dosage will have to be determined empirically, using a minimum initial dosage and repeating with additional peptide as required, especially since the effect is proportional to the peptide concentration, and will differ with different peptides and MHC-I proteins.

In contrast to the method for inducing self-destruction of CTL clones, the peptides are administered at discrete intervals of time to vaccinate an individual against the peptide. Since the goal in this case is not to induce self-destruction of the CTLs directed against the peptide, the peptide is administered at intervals such that the CTLs are no longer activated at the time of the subsequent exposure. Only cytolytically active cells are subject to self-lysis.

The present invention will be further understood with reference to the following non-limiting examples demonstrating selective

destruction of $CD8^+$ CTLs by administration of cognate peptides, both *in vitro* and *in vivo*, and inhibition of the destruction of the CTLs using non-cognate peptides that block presentation of the cognate peptide by an appropriate MHC-I protein.

Example 1: The Effect of Peptides on Cell Survival.

During attempts to elicit anti-influenza virus CTLs from a human lymphoid cell line (derived from peripheral blood) it was observed that the addition of an immunodominant influenza virus peptide (matrix protein peptide 253-289) led to a gradual disappearance of CD8 T cells and the persistence of CD4 +T cells. This observation was verified by following cell counts of cloned $CD8^+$ CTLs that were cultured in the absence or presence of cognate peptides at various concentrations.

The results are shown in Figure 1. Panels A and B, Fig. 1, show what happened when a cognate peptide (OVA-2) was added to an anti-ovalbumin CTL clone (2H7) that had been stimulated for the preceding three days by incubating it with specific target cells, irradiated EG7-OVA cells, which are EL-4 tumor cells transfected with the ovalbumin gene. Cell counts determined daily over the following 4 days showed that when no peptide was added (0, control in Fig. 1A), the number of CTL per ml was $1.4x10^6$ on day 3 and $2x10^6$ on day 4. However, in cultures to which the OVA-2 peptide was added at 20 μg/ml, the number of CTL per ml dropped to $0.2x10^6$ on day 3; on day 4 there were too few cells to count ($< 0.1x10^6$). The magnitude of the decline was proportional to the concentration of peptide added.

In a parallel experiment with a tumor-specific CTL clone (12D3), one day after transferring the target cell-stimulated CTLs into fresh medium the number of cells per ml were $2.2x10^6$ in the absence

of the added cognate peptide, T1 (as shown in Fig. 1C), and had fallen to around $1x10^5$ after adding T1 at 10 μg per ml. The magnitude of the decline was also proportional to the concentration of the peptide.

Similar effects were seen when another anti-ovalbumin CTL clone, 3G12, was stimulated for three days with the EG6-OVA cells and then separated from the latter by dilution into fresh medium to which the OVA-2 peptide was added. As shown by Fig. 2D, one day later the number of 3G12 cells per ml was 2.85×10^6 when no peptide had been added (0, control) and was almost 10-fold lower when OVA-2 was added at 20 μg/ml.

Example 2: Effect of Peptides on Cytolytic Activity.

The effect of peptides on cytolytic activity was then examined. After maintaining three clones for three days in the presence or absence of various peptides, they were tested for cytolytic activity. The results are shown in Figure 3. When no peptide had been added (Fig. 3, column at extreme left), the cytolytic activity observed corresponded to the clones' specificity. Virtually all anti-ovalbumin CTLs that are raised in C57BL/6 mice (H-2^b) by the immunization program used to derive clone 4G3 have been shown to recognize the OVA-2 peptide in association with K^b. This specificity (OVA-2/K^b) is in agreement with the cytolytic activity of clone 4G3 (left, uppermost panel, Fig. 3) and the other anti-OVA CTL clones examined. Similarly, the cytolytic activities of clones 12D3 and IE1 are in agreement with previous studies: 12D3 is specific for the T1 peptide in association with L^d and IE1 is known to be specific for a peptide in the pp89 protein of mouse cytomegalovirus (MCMV) in association with L^d.

When each of these clones was incubated for three days with each of the peptides and then tested for cytotoxic activity, it was evident

that only a clone's cognate peptide eliminated that clone's activity: the OVA-2 peptide inactivated only clone 4G3, T1 inactivated only clone 12D3, and pp89 inactivated only clone IE1. The noncognate peptides tested had no effect.

Example 3: Cognate Peptide Induced Self-Destruction of CTLs Can Be Inhibited by a Peptide That Blocks Lysis of a Conventional Target Cell.

By screening around twenty unrelated peptides it was found that two of them blocked sensitization of EL-4 cells by the OVA-2 peptide for lysis by anti-ovalbumin CTLs. Considerable evidence indicates that such selectively inhibiting peptides compete with the cognate peptide's presentation by the target cells' MHC-I protein. Figure 4 shows that an inhibiting peptide (pp89) also blocked the OVA-2-induced destruction of an anti-ovalbumin CTL clone (4G3 cells). The extent of inhibition varied with the blocking peptide's concentration. Thus, in undergoing self-destruction these CTLs evidently recognized the cognate peptide on themselves in the same way as on conventional target cells.

Independent experiments strongly suggest that (1) the inhibiting peptide competes with the cognate peptide for binding to the MHC-I protein involved (K^b), and (2) the inhibiting peptide/K^b complex is not recognized by 4G3 cells' antigen-specific receptor.

Example 4: Inactivation of CTLs *in vivo* with cognate peptides.

To measure the cytolytic activity of CTLs to many antigens, it is necessary to obtain activated precursors ("primed") CTLs from animals that have been immunized with the antigen in appropriate form, and to restimulate these cells in culture. If a cognate peptide acts on CTLs *in vivo*, as it does *in vitro* (Figures 2-4), then injecting the immunized animals with the peptide, at an appropriate time, would be

expected to eliminate or substantially reduce the level of cytolytic activity that is subsequently measurable *in vitro*.

To measure anti-ovalbumin CTL activity, isolated spleen cells were stimulated one or more times in culture with irradiated EG7-OVA cells, and then tested for cytolytic activity on three target cells: 1) EG7-OVA, 2) EL-4 cells that were sensitized by incubating them with the OVA-2 peptide (termed EL-4 + OVA-2 targets), and 3) EL-4 cells alone (termed nonsensitized targets). Greater lysis of the EL-4 + OVA-2 targets than of nonsensitized EL-4 targets provided a measure of OVA-2-specific CTLs.

Spleen cells that were derived from naive mice and stimulated only *in vitro* had no cytolytic activity against OVA-2, although they did manifest some low-level cytolytic activity against some epitopes of EL-4 cells, as shown by Fig. 5, panels A1 and A2. In contrast, anti-OVA-2 activity was pronounced in spleen cells obtained from mice that had been primed with EG7-OVA cells (Fig. 5, panels B1 and B2), or with ovalbumin (Fig. 5, panels C1 and D1), and even to some extent with the OVA-2 peptide alone (Fig. 5, panels C2 and D2).

To determine if an injection of the cognate peptide (OVA-2) could decrease the effect of priming the CTLs *in vivo*, the peptide was injected once into each of several immunized mice, from 1 to 5 days after the final priming injection with ovalbumin. The results are shown in Fig. 6. Mice that had received no peptide at all, or had been injected with the peptide on day 1 or day 5 after the final priming injection with ovalbumin, had indistinguishable levels of OVA-2 specific CTL activity (see none, day 2 and day 5, Fig. 6). However, injection of the peptide on day 2 resulted in the elimination of OVA-2 specific cytolytic activity; the remaining activity was essentially the same on all

three target cells, and thus, under these circumstances, the active CTLs were specific for epitopes of the EL-4 cells but not for OVA-2. Injection of peptide on day 3 or day 4 resulted in a reduction but not in a complete elimination of anti-OVA-2 activity.

Different protocols are shown in Fig. 7, which also shows that primed CTLs can be eliminated *in vivo* by their cognate peptide. In one (panels A1 and A2) the peptide was injected once 14 days after an initial priming injection with EG7-OVA and 16 days before a second priming with these cells. The first assay of spleen cells, after five days in culture, showed activity only against EL4 epitopes, not against OVA-2 (Fig. 7, A1), but the second assay, performed after additional stimulation with EG7-OVA cells revealed distinct CTL activity against OVA-2 and virtually none against EL-4 (Fig. 7, A2). While the injected peptide in this situation may have partly inactivated primed anti-OVA-2 CTLs, it did not eliminate them completely. In the protocol illustrated in Panels B1 and B2 of Fig. 7, the OVA-2 peptide was injected twice, the second time four days after a booster injection of EG7-OVA. In this situation no anti-OVA-2 CTL activity was detected after the first or second spleen cell assay, although in the second assay, performed after repeated stimulation with EG7-OVA cells, cytolytic activity directed against epitopes of EL4 was evident (panel B2, Fig. 7). Hence, with this schedule of injections the cognate peptide appears to have successfully eliminated primed CTLs from the immunized mice.

All of these results, taken together demonstrate that 1) a cognate peptide can specifically inactivate the corresponding CTLs *in vivo*, and 2) the inactivation probably occurs when the peptide achieves a critical concentration for a critical period in the immediate vicinity of cells that

are cytolytically active: administration of the peptide at a time when the cells were probably inactive had no discernible effect.

Example 5: Destruction of CTLs Induced by Cognate Peptides is Apparently Due to Suicide, rather than Fratricide.

Because of clone IE1's apparent lack of resistance to cytolytic attack by CTLs, these cells were used to ask whether their destruction by their cognate peptide comes about because the cells attack each other (fratricide) or because individual cells are induced to undergo self-destruction (suicide). ^{51}Cr-labeled IE1 cells were incubated with their cognate peptide at varying cell densities, achieved by incubating the same number of cells in different volumes of culture medium and by incubating various numbers of cells in the same volume. The specific release of ^{51}Cr was then measured after 5 hours, in a conventional assay for CTL activity, and again after an overnight incubation. After 5 hrs the lysis of IE1 cells was slightly greater when the cells were more closely packed, suggesting that CTL-CTL interactions are involved in the lysis of these cells. However, after overnight incubation the amount of specific ^{51}Cr release was independent of initial cell density, as though the peptides induced individual cells to kill themselves.

For an independent approach to this question, CTLs were also examined in the light microscope. Upon adding the cognate (OVA-2) peptide to 4G3 (anti-ovalbumin) CTLs, most of the cells underwent a striking change after around one hour: they became small, round, and highly refractile, in contrast to the highly irregular shape of normal CTLs. By the next morning most of the cells were dead, suggesting that the small round cells seen after one hour were already doomed.

This striking change was clearly delayed by including in the medium an inhibiting peptide (pp89) at a 20-fold molar excess over the

cognate peptide. The speed with which the cognate peptide induced an effect suggested that the individual CTL could recognize and respond to itself, a prerequisite for suicide, rather than because CTL-CTL interactions were required. Nevertheless, to reduce the frequency of cell-cell contacts, dextran at 10% was included in the medium to increase its viscosity and thereby reduce cell movement. Under these circumstances cell motility was greatly reduced, although not entirely eliminated.

Even though a role for cell-cell interactions cannot be fully discounted, all of the evidence, taken together, suggests that the cognate-peptide elicits its specific effect primarily because it induces self-destruction of individual CTLs.

The ease with which CTL self-destruction can be elicited by the cognate peptide provides the basis for simplified procedures for screening peptides, with CTLs, in order to identify both the suicide-inducing cognate peptides as well as those other peptides that specifically prevent CTL self-destruction by competively binding to the same MHC-I as the cognate peptide.

Modifications and variations of the present invention, a method for inducing self-lysis of targeted CTLs by activating the CTLs and exposing them to their cognate peptides, will be obvious to those skilled in the invention from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

We claim:

1. A method for modulating selective destruction of cytotoxic thymus derived lymphocytes comprising exposing the lymphocytes to a concentration of between nanograms and micrograms per milliliter of the cognate peptide for the lymphocytes.

2. The method of claim 1 wherein the lymphocytes are *in vivo*.

3. The method of claim 1 wherein the lymphocytes are *in vitro*.

4. The method of claim 1 wherein the peptide is a linear amino acid sequence of between 5 and 50 amino acids.

5. The method of claim 1 wherein the peptide is a part of self-antigen or viral antigen.

6. The method of claim 1 wherein the peptide is administered in microgram to milligram quantities.

7. The method of claim 2 wherein the peptide is in a pharmaceutically acceptable carrier for administration to a patient.

8. The method of claim 7 wherein the peptide is encapsulated in a polymeric matrix suitable for controlled release.

9. The method of claim 8 wherein the polymer is selected from the group consisting of ethylenevinyl acetate, poly(lactic acid), poly(glycolic acid), polyanhydrides, poly(ortho esters), polyesters, proteins, blends and copolymers thereof.

10. The method of claim 5 wherein the patient has an autoimmune disorder.

11. The method of claim 5 wherein the patient has a viral infection and the pathology is augmented by anti-viral cytotoxic T lymphocytes.

12. The method of claim 1 wherein the lymphocytes are CD8+ lymphocytes.

13. The method of claim 1 wherein the lymphocytes are human cytolytic CD4+ lymphocytes.

14. The method of claim 1 wherein the lymphocytes are cytolytically activated at the time of exposure to the peptides.

15. The method of claim 1 wherein the peptides are administered after or in combination with an immunostimulant selected from the group consisting of adjuvants and lymphokines.

16. The method of claim 1 wherein the lymphocytes are exposed to the peptides for at least six hours.

17. The method of claim 16 wherein the lymphocytes are exposed to the peptides until all the lymphocytes recognizing the peptides are dead.

18. The method of claim 5 wherein the cognate peptides have the same sequence as a critical segment of the thyroid stimulating hormone receptor.

19. The method of claim 5 wherein the cognate peptides have the same sequence as a critical segment of the acetylcholine receptor.

20. A method for inducing proliferation of cytotoxic thymus derived lymphocytes comprising administering between nanograms and micrograms of peptide per milliliter to unactivated cytolytic lymphocyte precursors.

21. The method of claim 20 wherein the lymphocytes are exposed to the peptides at discrete intervals over time, when the lymphocytes recognizing the peptides are not cytolytically activated.

22. The method of claim 20 wherein the peptides is a linear sequence of between 5 and 50 amino acids.

23. The method of claim 20 wherein the peptides are administered in combination with a pharmaceutically acceptable carrier which does not prolong exposure of the lymphocytes to a high concentration of the peptides for more than six hours.

24. A method for determining the cognate peptide for cytotoxic thymus derived lymphocytes comprising isolating cytotoxic lymphocytes, exposing them to between nanograms and micrograms per milliliter of a peptide consisting of between 5 and 50 amino acids and determining whether the peptide induces self-destruction of the cytotoxic lymphocytes.

25. The method of claim 24 wherein the lymphocytes are isolated from tissue involved in an autoimmune or viral inflammatory response.

26. The method of claim 24 wherein the peptides are derived from MHC proteins from the surface of cells involved in an autoimmune or viral inflammatory response.

27. A method for determining the cognate peptide for cytotoxic thymus derived lymphocytes comprising isolating cytotoxic lymphocytes, exposing them to between anograms and micrograms per milliliter of a peptide consisting of between 5 and 50 amino acids and determining whether the peptide induces a shape change in the cytotoxic lymphocytes.

28. The method of claim 27 further comprising determining whether the peptide induces self-destruction of the cytotoxic lymphocytes.

29. The method of claim 27 wherein the lymphocytes are isolated from tissue involved in an autoimmune or viral inflammatory response.

30. The method of claim 25 wherein the shape change is determined by microscopic examination of the cytotoxic lymphocytes.

FIGURE 1a

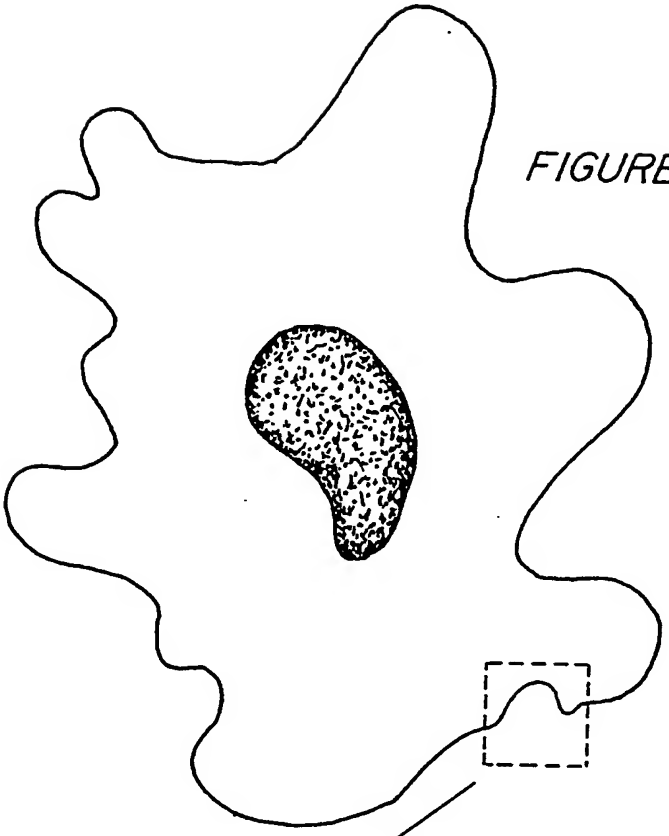
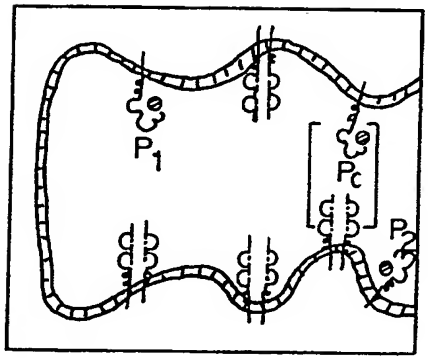
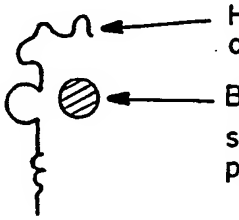


FIGURE 1b

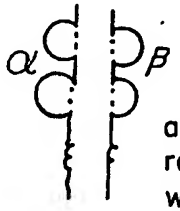




Heavy chain subunit of MHC-I protein

B_2 microgloblin (light subunit of MHC-I protein)

P = peptide (P_1, P_2, etc; P_c = cognate peptide)



antigen - specific T cell receptor (a heterodimer with an α and a β subunit)

FIGURE 2a

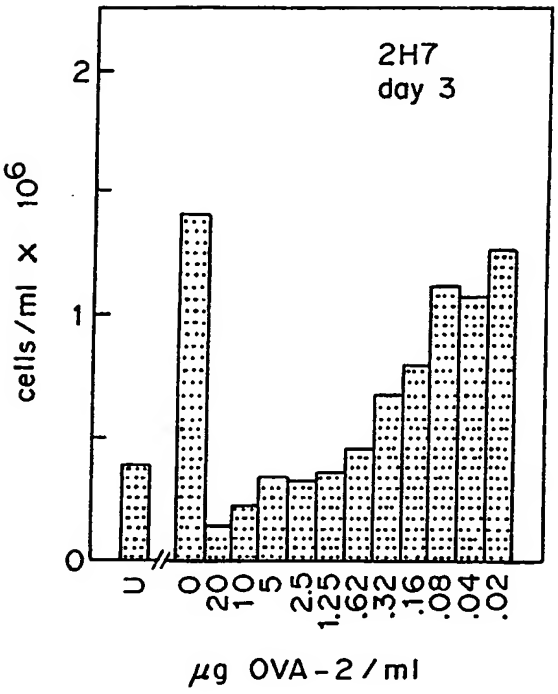


FIGURE 2b

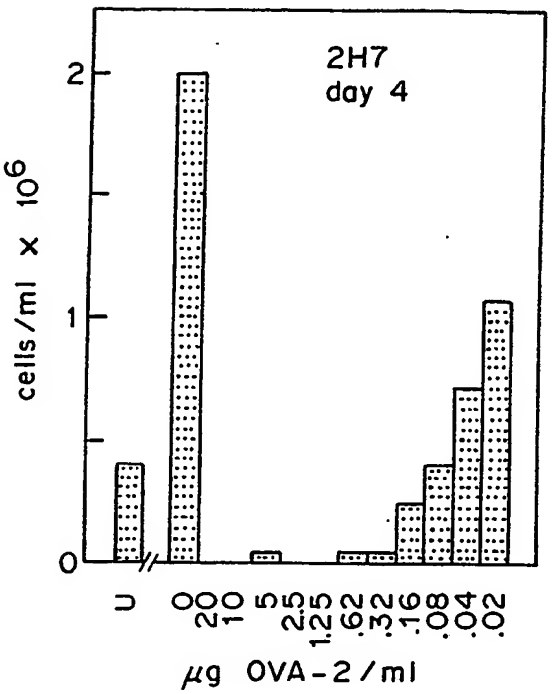


FIGURE 2c

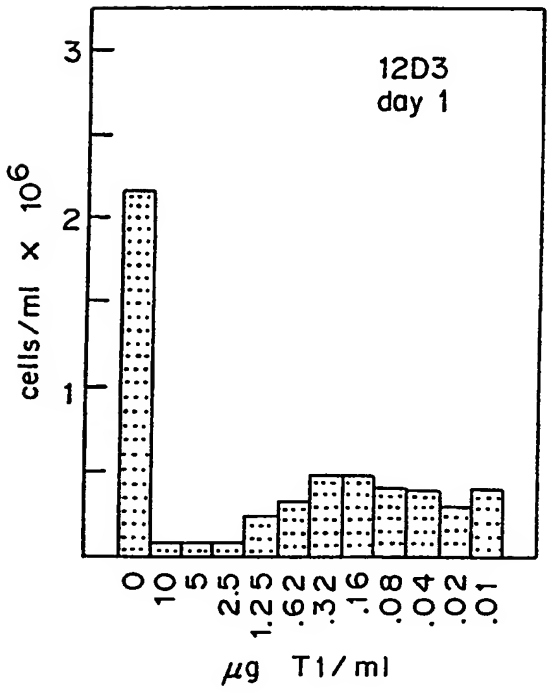


FIGURE 2d

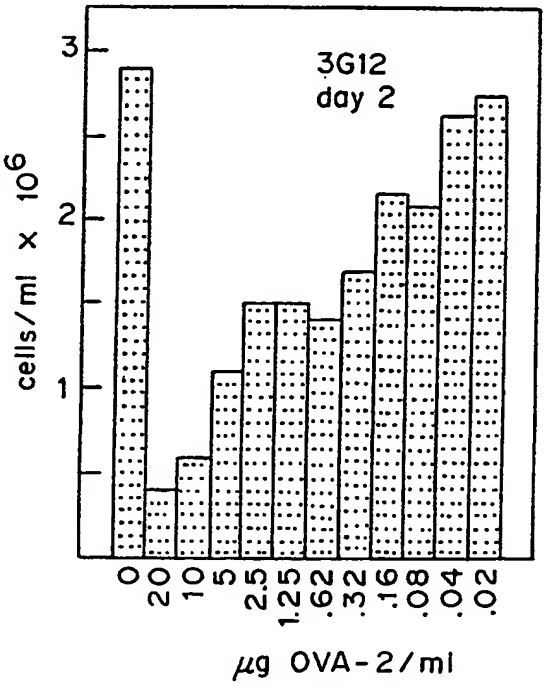


FIGURE 3

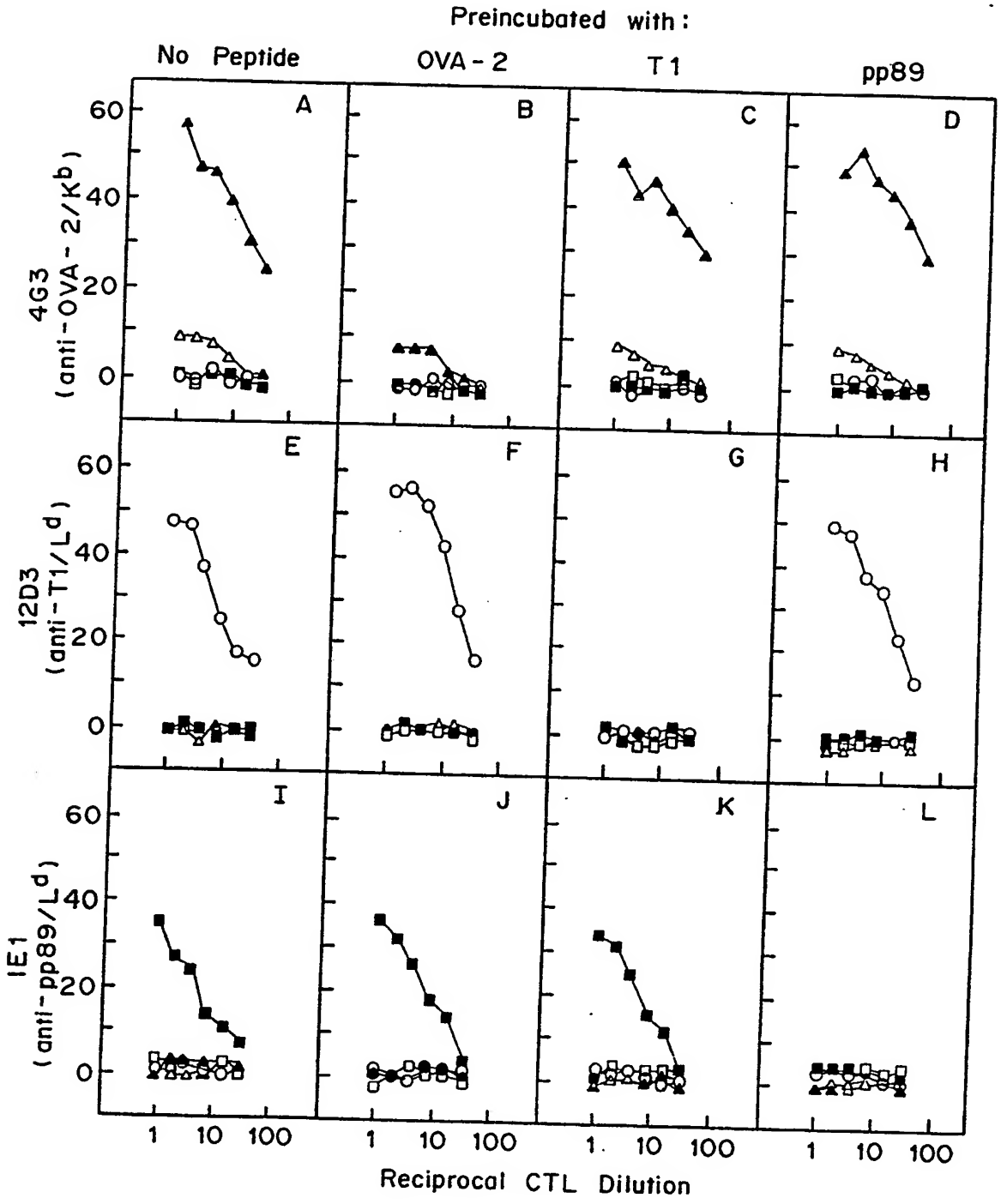

Preincubated with:
No Peptide
OVA - 2
T1
pp89
A
B
C
D
E
F
G
H
I
J
K
L
4G3 (anti-OVA - 2/K^b)
12D3 (anti-T1/L^d)
IE1 (anti-pp89/L^d)
60
40
20
0
1
10
100
Reciprocal CTL Dilution

FIGURE 4

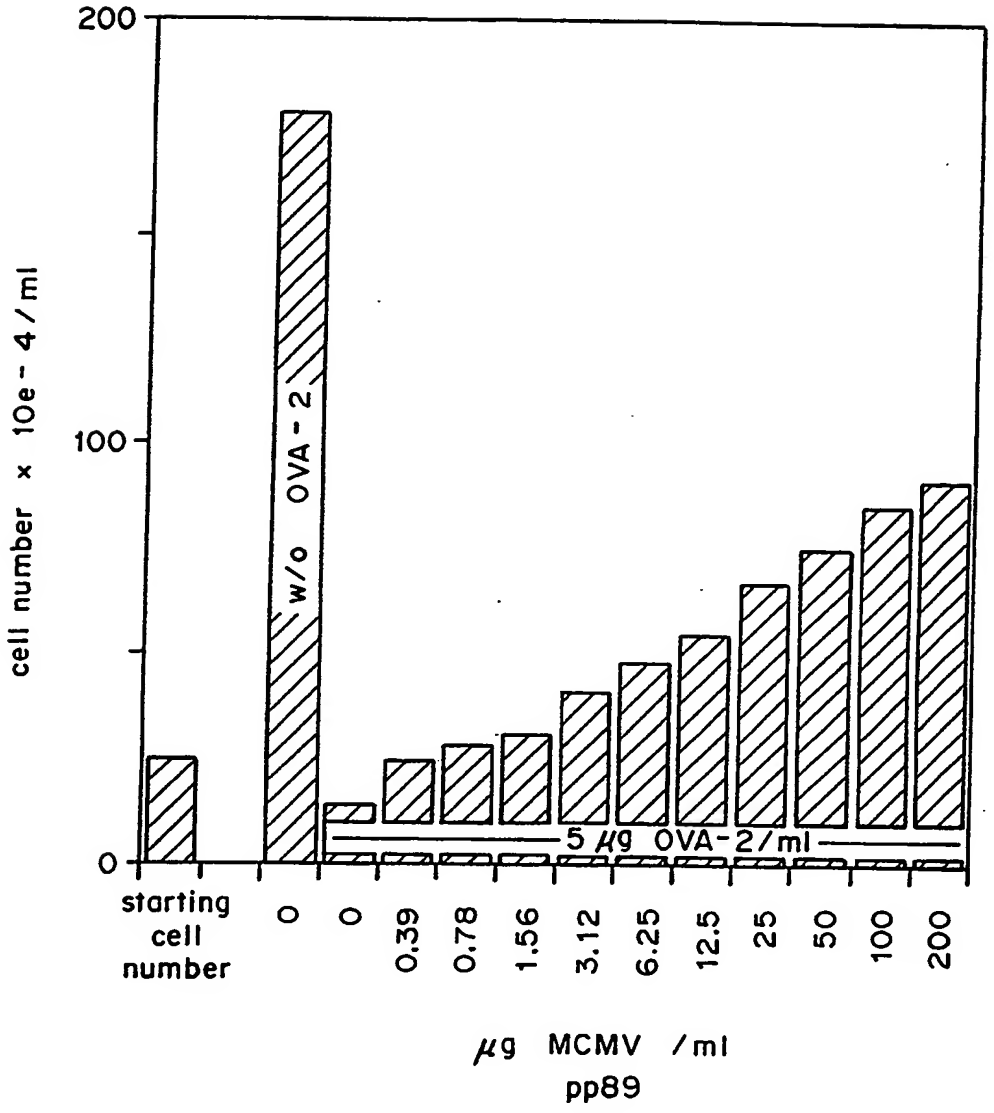

200
100
0
cell number x 10e-4/ml
w/o OVA-2
5 µg OVA-2/ml
starting cell number
0
0
0.39
0.78
1.56
3.12
6.25
12.5
25
50
100
200
µg MCMV /ml pp89

FIGURE 5

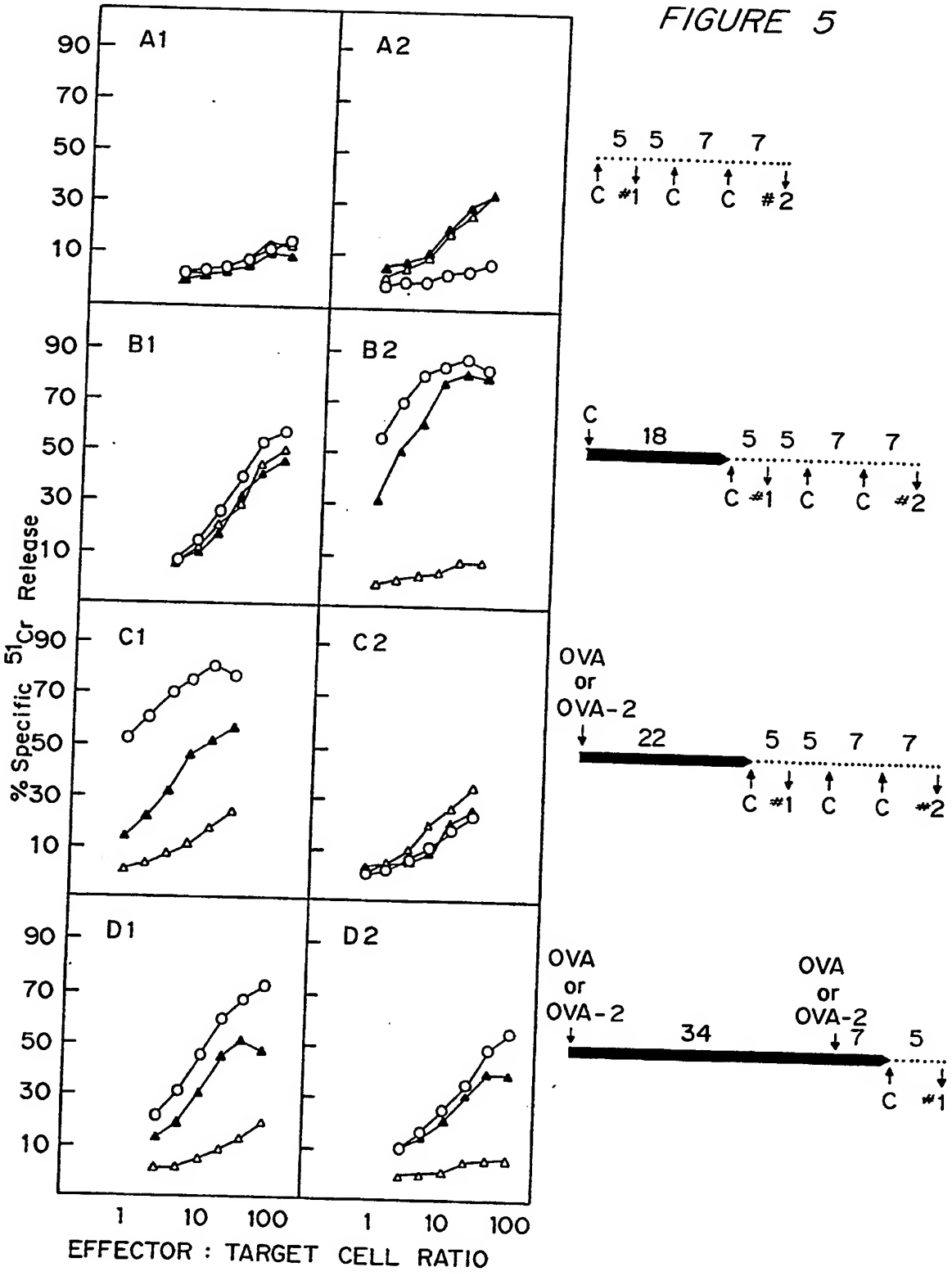

A1
A2
B1
B2
C1
C2
D1
D2
90
70
50
30
10
% Specific 51Cr Release
1
10
100
EFFECTOR : TARGET CELL RATIO
5 5 7 7
C #1 C C #2
C
18
5 5 7 7
C #1 C C #2
OVA
or
OVA-2
22
5 5 7 7
C #1 C C #2
OVA
or
OVA-2
34
OVA
or
OVA-2
7
5
C #1

FIGURE 6a

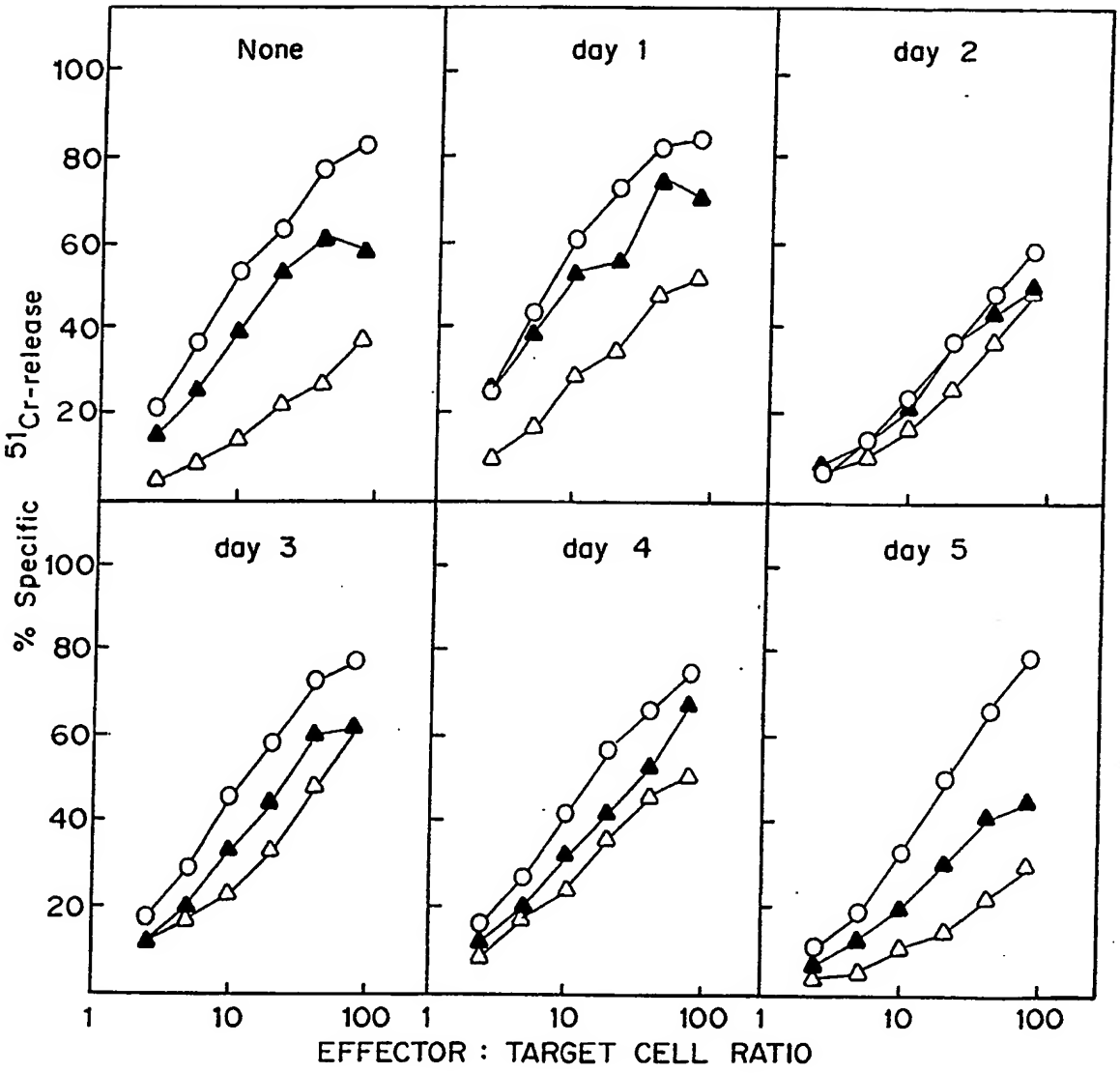


FIGURE 6b

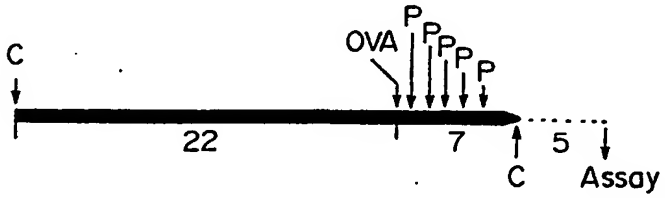
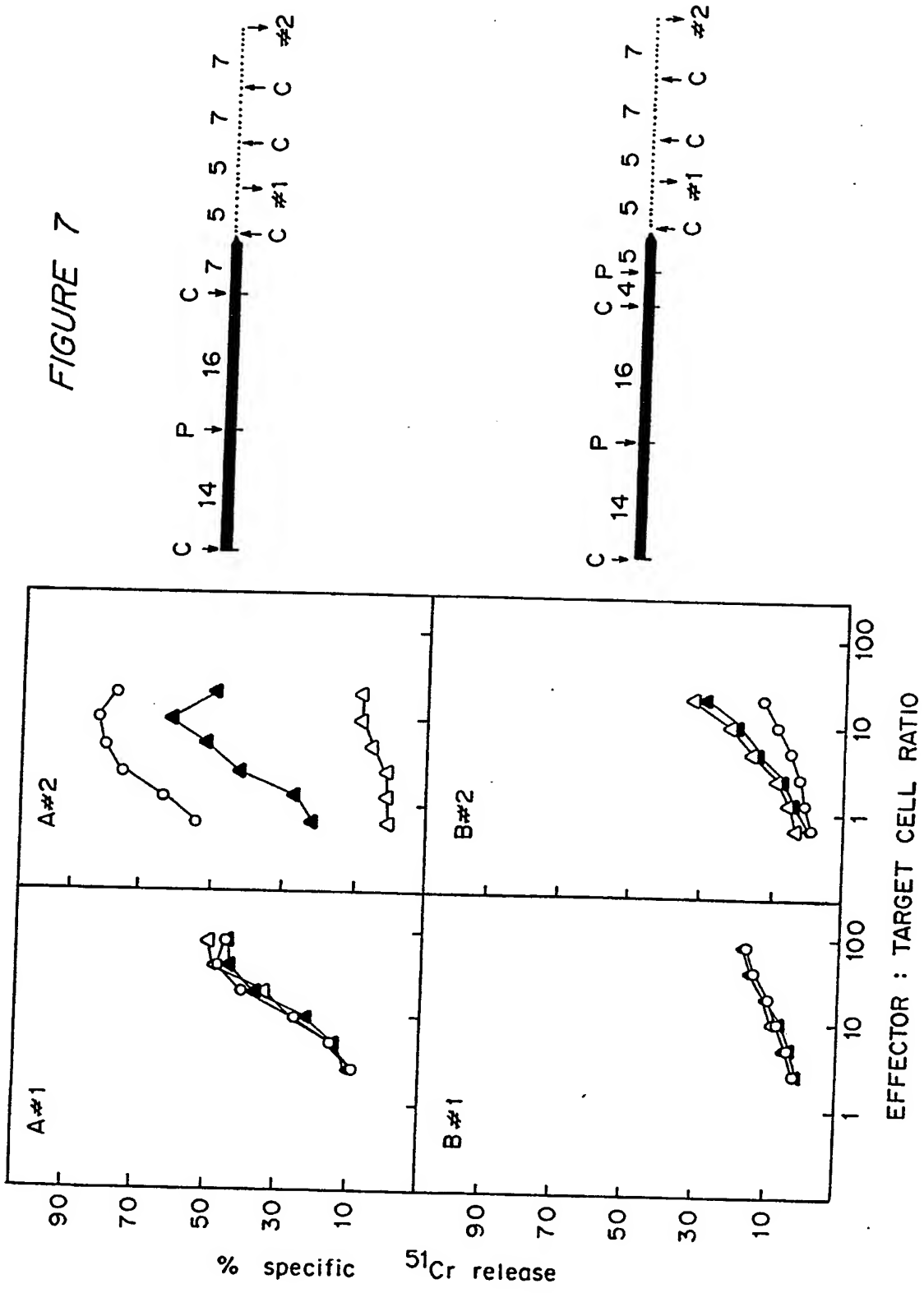


FIGURE 7


C 14 P 16 C 7 5 7 C #1 C C #2
C 14 P 16 C P 4 5 5 7 C #1 C C #2
A #2
B #2
A #1
B #1
90 70 50 30 10
% specific ^{51}Cr release
1 10 100
EFFECTOR : TARGET CELL RATIO

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03954

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 45/05, 47/00, 39/12

US CL: 424/86, 88, 89; 435/240, 4

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/86; 435/240; 424/88; 435/4; 424/89;

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Dialog (Files 5 399, 357, 154, 155, 72), USPQ Automated Patent Systems (File USPAT 1971-1991).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,861,589 (JU) 29 August 1989, see entire document.	1-30
Y	Proceedings National Academy of Science, volume 74, No. 5, issued May 1977, Bevan et al., "Killer Cells reactive to altered-self antigens can also be alloreactive", pages 2094-2096, see entire document.	1-30
Y	Journal of Experimental Medicine, volume 169, issued March 1989, Carbone et al., "Induction of Ovalbumin-specific Cytotoxic T cells by in vivo peptide immunization", pages 603-612, page 603.	1-30

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05 August 1991	26 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Lynette F. Smith

Form PCT/ISA/210 (second sheet) (Rev.11-87)